# Reciprocal Interaction of the Circadian Clock with the Iron Homeostasis Network in Arabidopsis[1][W][OA]

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In plants, iron (Fe) uptake and homeostasis are critical for survival, and these processes are tightly regulated at the transcriptional and posttranscriptional levels. Circadian clocks are endogenous oscillating mechanisms that allow an organism to anticipate environmental changes to coordinate biological processes both with one another and with the environmental day/night cycle. The plant circadian clock controls many physiological processes through rhythmic expression of transcripts. In this study, we examined the expression of three Fe homeostasis genes (*IRON REGULATED TRANSPORTER1* [*IRT1*], *BASIC HELIX LOOP HELIX39*, and *FERRITIN1*) in Arabidopsis (*Arabidopsis thaliana*) using *promoter:LUCIFERASE* transgenic lines. Each of these promoters showed circadian regulation of transcription. The circadian clock monitors a number of clock outputs and uses these outputs as inputs to modulate clock function. We show that this is also true for Fe status. Fe deficiency results in a lengthened circadian period. We interrogated mutants impaired in the Fe homeostasis response, including *irt1-1*, which lacks the major high-affinity Fe transporter, and *fit-2*, which lacks Fe deficiency-induced TRANSCRIPTION FACTOR1, a basic helix-loop-helix transcription factor necessary for induction of the Fe deficiency response. Both mutants exhibit symptoms of Fe deficiency, including lengthened circadian period. To determine which components are involved in this cross talk between the circadian and Fe homeostasis networks, we tested clock- or Fe homeostasis-related mutants. Mutants defective in specific clock gene components were resistant to the change in period length under different Fe conditions observed in the wild type, suggesting that these mutants are impaired in cross talk between Fe homeostasis and the circadian clock.

The rotation of the earth on its axis means that life has evolved on a world characterized by dramatic, recurrent, and rhythmic environmental change. Considerable evidence has accumulated in support of the hypothesis that the ability to measure and use time to coordinate biology with the environment in anticipation of coming change confers a fitness advantage (Ouyang et al., 1998; Resco et al., 2009; Yerushalmi and Green, 2009). Thus, circadian rhythms have been described in organisms from all domains of life, including bacteria (Mackey et al., 2011) and archaea (Whitehead et al., 2009; Edgar et al., 2012), as well as eucarya, including plants and animals (Lowrey and Takahashi, 2011; McClung, 2011; Zhang et al., 2011).

Circadian rhythms, the subset of rhythms with a period of approximately 24 h, are generated by an endogenous circadian clock. The fitness advantage conferred by the circadian clock emerges from its regulation of many aspects of biology, including basic metabolism, hormone signaling, and responses to biotic and abiotic stress (Doherty and Kay, 2010; Wang et al., 2011; Sahar and Sassone-Corsi, 2012). In plants, the circadian clock is emerging as a key player in the coordination of metabolism and growth (Dodd et al., 2005; Nozue et al., 2007; Gutiérrez et al., 2008; Michael et al., 2008a; Fukushima et al., 2009; Graf et al., 2010; Kerwin et al., 2011; Kunihiro et al., 2011; Nozue et al., 2011). One major mechanism by which the clock coordinates so many pathways and processes is via pervasive control of gene expression at the levels of transcription, transcript processing, and transcript abundance (Covington et al., 2008; Doherty and Kay, 2010; Filichkin et al., 2010; Sanchez et al., 2010). It is becoming increasingly clear that in plants, clock function not only regulates many aspects of cellular metabolism and physiology (Harmer, 2009; McClung and Gutiérrez, 2010; Pruneda-Paz and Kay, 2010; McClung, 2011), including solute transport (Haydon et al., 2011), but is, in turn, modulated by the cellular metabolic state. Plant clock function has been shown to respond to the uptake and homeostasis of Suc (Bläsing et al., 2005; Knight et al., 2008; Dalchau et al., 2011) and other nutrients, including magnesium (Mg; Hermans et al., 2010), copper (Cu; Andrés-Colás et al., 2010; Peñarrubia et al., 2010), and nitrogen (N; Gutiérrez et al., 2008). Similarly, clock function is highly responsive to metabolic state, including redox status, in cyanobacteria (Ivleva et al., 2005; Rust et al., 2011) and

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mammals (Rutter et al., 2002; Asher and Schibler, 2011; Bass, 2012).

The Arabidopsis (*Arabidopsis thaliana*) circadian clock is a complex network of interlocked feedback loops. Central to the clock is a feedback loop consisting of two Myb transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), with a transcriptional repressor, TIMING OF CAB EXPRESSION1 (TOC1), of the PSEUDO-RESPONSE REGULATOR (PRR) family (McClung, 2011; Nagel and Kay, 2012). Much of the regulation of clock components is transcriptional, but this is augmented with considerable posttranscriptional (Staiger and Green, 2011) and posttranslational (McClung, 2011; Nagel and Kay, 2012) regulation. Many clock components, including CCA1, LHY, and TOC1, are differentially phosphorylated throughout the circadian cycle, and this posttranslational modification alters protein activity and, in some cases, protein stability (Kusakina and Dodd, 2012). In particular, an F-box protein, ZEITLUPE (ZTL), targets TOC1 and a second PRR protein, PRR5, for ubiquitylation and subsequent proteasomal degradation (Más et al., 2003; Kiba et al., 2007; Fujiwara et al., 2008).

Plants carry out oxygenic photosynthesis and aerobic respiration, both of which require iron (Fe) and generate toxic reactive oxygen species (ROS). The circadian clock regulates photosynthetic activity at the cellular, organismal, and ecosystem levels (Hennessey and Field, 1991; Salomé et al., 2002; Resco de Dios et al., 2012). Not surprisingly, the production of ROS varies throughout the circadian day, and the circadian clock is emerging as a key regulator of cellular ROS-responsive pathways (Edgar et al., 2012; Lai et al., 2012). Principal sources of ROS include the electron transport chains associated with both photosynthesis and respiration. Fe-containing proteins are key components of these electron transport chains, because Fe can exist in multiple redox states, serving as either electron acceptor or donor. The redox-active nature of Fe allows it to generate ROS via the Fenton reaction, in which Fe(II) reacts with hydrogen peroxide to yield hydroxyl free radicals (Halliwell and Gutteridge, 1992). Thus, Fe is a necessary micronutrient, yet its levels must be tightly controlled to prevent the accumulation of damaging levels of ROS.

Fe homeostasis is imposed at multiple levels. First, Fe acquisition is typically limiting because Fe is only sparingly soluble at neutral pH in aerobic conditions (Palmer and Guerinot, 2009). Dicots and some monocots, although not the grasses, apply a tripartite response to assimilate Fe, and each of these three responses is induced in response to Fe limitation. These plants acidify the soil by proton extrusion to increase Fe solubility, reduce Fe from the ferric [Fe(III)] to the ferrous [Fe(II)] form, and then take up ferrous Fe via high-affinity Fe(II) transporters. In Arabidopsis, $H^+$-ATPases of the AHA (for Arabidopsis $H^+$ ATPase) family are likely responsible for the proton extrusion (Santi and Schmidt, 2009), the ferric chelate reductase FERRIC REDUCTION OXYGENASE2 (FRO2) reduces Fe(III) to Fe(II) (Robinson et al., 1999), and the high-affinity Fe(II) transporter IRON-REGULATED TRANSPORTER1 (IRT1) takes up Fe into the root epidermis (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). mRNAs for several *AHA* genes, *FRO2*, and *IRT1* accumulate only under Fe limitation (Palmer and Guerinot, 2009). Second, within the plant, Fe is sequestered in the vacuole (Kim et al., 2006) or stored in ferritin nanocages (Palmer and Guerinot, 2009). *FERRITIN* (*FER*) gene expression is induced by Fe sufficiency (Gaymard et al., 1996; Petit et al., 2001).

In plants, the diurnal rhythm in photosynthetic activity confers a rhythm in the generation of ROS (Lai et al., 2012). Because photosynthetic activity is under circadian control and because the circadian clock is emerging as a central player in the control of levels of ROS (Edgar et al., 2012; Lai et al., 2012), we wished to determine whether the circadian clock might regulate the expression of Fe homeostasis genes. Such a link was suggested by the identification of *TIME FOR COFFEE* (*TIC*), which encodes a nuclear circadian clock component (Ding et al., 2007), as a regulator of Fe overload-responsive genes, including *FER1* (Duc et al., 2009). We examined the transcription and mRNA accumulation of three key Fe homeostasis genes, *IRT1*, the basic helix-loop-helix transcription factor gene *basic Helix-Loop-Helix39* (*bHLH39*; Vorwieger et al., 2007; Wang et al., 2007, 2013), and *FER1*. *IRT1* and *bHLH39* are critical elements of the Fe acquisition mechanism and are both induced by Fe deficiency, whereas *FER1* encodes a key Fe storage protein and is induced by Fe sufficiency. We observed that the circadian clock regulates the transcription and mRNA accumulation of each of these three Fe homeostasis genes. In addition, we showed that mutants known to affect clock function also affected the circadian period length of expression of these Fe homeostasis genes. Finally, we also showed that the Fe status of the plants affected circadian period length, indicating that the Fe homeostasis network is not only an output of the circadian clock but that Fe status is a nutritional input that modulates the pace of the clock.

## RESULTS

### The Plant Circadian Clock Regulates the Expression of Fe Homeostasis Genes

We wished to determine whether key elements of the Fe homeostasis network were under the control of the circadian clock. Although mRNA abundance of *IRT1* and *FRO2* had earlier been shown to cycle in plants growing in light/dark (LD) cycles (Vert et al., 2003), this cycling had not been shown to persist in continuous conditions and so had not been established to be under circadian clock control. The transcript abundance of *FER1* has been shown to cycle in both LD and continuous light (LL; Duc et al., 2009). We first

interrogated the DIURNAL database (Mockler et al., 2007) and confirmed that steady-state mRNA abundance for three representative Fe homeostasis genes, *IRT1*, *bHLH39*, and *FER1*, oscillated in LD or warm/cold environmental cycles (Supplemental Fig. S1A). We generated transgenic Arabidopsis lines in the Columbia-0 (Col-0) background carrying gene fusions in which the promoters of *IRT1*, *bHLH39*, or *FER1* were used to drive the expression of the firefly *LUCIFERASE* (*LUC*) gene. Expression of each *promoter*:*LUC* (*Pro*:*LUC*) fusion transgene recapitulated the spatial patterns previously described on the basis of transcript accumulation (Supplemental Fig. S2). Expression of *ProIRT1*:*LUC* was detected only in roots of Fe-deficient but not in Fe-replete roots or in shoots regardless of Fe status (Eide et al., 1996). Expression of *ProbHLH39*:*LUC* was detected in both shoots and roots (Vorwieger et al., 2007; Wang et al., 2007). Expression of *ProFER1*:*LUC* was detected primarily in shoots but weakly in roots (Gaymard et al., 1996). Expression of *ProIRT1*:*LUC* and of *ProbHLH39*:*LUC* was greater at Zeitgeber time (ZT)48 than at ZT36 (where ZT0 is dawn), whereas expression of *ProFER1*:*LUC* showed the opposite pattern (Supplemental Fig. S2), consistent with the temporal expression patterns shown in Supplemental Figure S1A. We measured LUC activity in seedlings growing in LD cycles and observed robust daily cycling of promoter activity (Supplemental Fig. S1B). To test whether the transcription of these three genes was independent of environmental cycles and, hence, under circadian control, we measured LUC activity in seedlings entrained in LD and released into continuous conditions (LL and constant temperature). Transcription of each *Pro*:*LUC* fusion cycled in LL with periods that were statistically indistinguishable from those of *ProCCA1*:*LUC* and *ProTOC1*:*LUC* (Fig. 1, A–C) and, hence, was clock regulated. We confirmed circadian oscillation of the abundance for each of the *IRT1*, *bHLH39*, and *FER1* transcripts in LL by quantitative reverse transcription (qRT)-PCR (Fig. 1D).

To confirm that the circadian clock regulates the expression of *IRT1*, we asked whether the period of

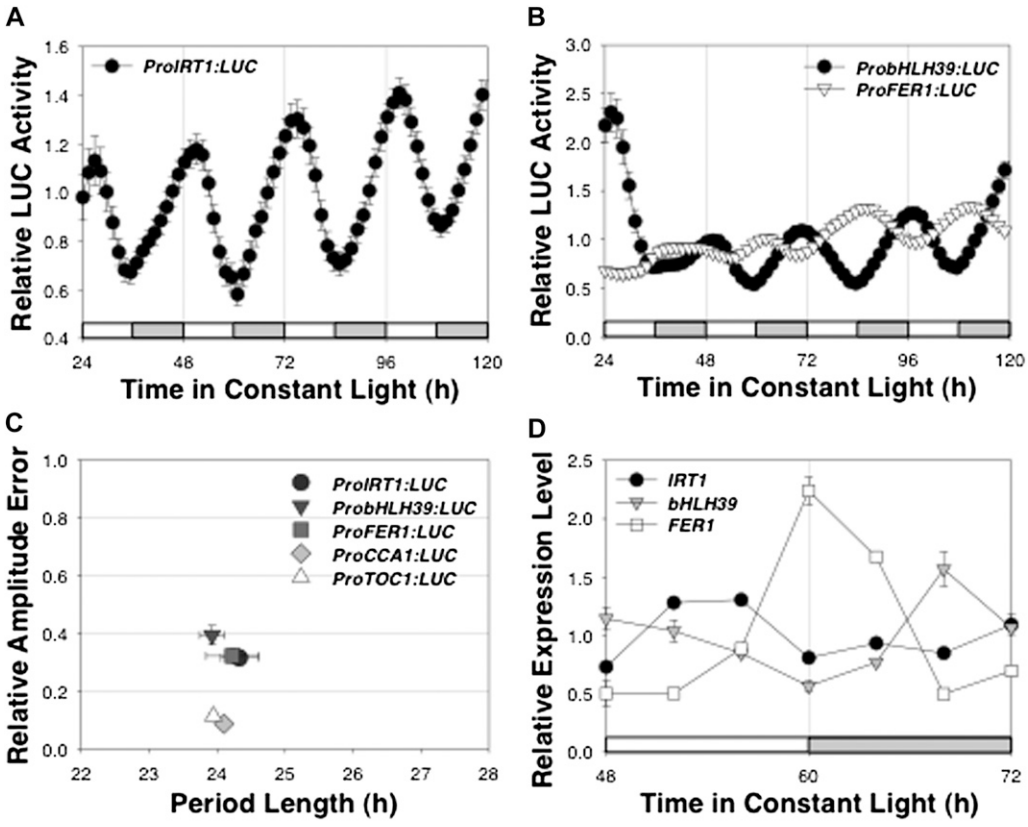


**Figure 1.** Circadian regulation of Fe homeostasis gene expression. A and B, Seedlings growing on minimal medium were entrained to photocycles (LD 12/12 h) for 6 d before release in LL. Average traces (mean ± SE, $n = 24$) are shown for luciferase activity of *ProIRT1*:*LUC* (A) and *ProbHLH39*:*LUC* and *ProFER1*:*LUC* (B) expression, normalized to the average activity over the duration of the experiment. C, Period versus relative amplitude error (RAE) for Fe homeostasis (*IRT1*, *bHLH39*, and *FER1*) and clock (*CCA1* and *TOC1*) gene expression. RAE is a measure of the strength of the oscillation, with RAE = 0 corresponding to a perfect sine wave and RAE = 1 defining the lower limit of statistically significant rhythmicity. D, Transcript levels (from two independent experiments) of *IRT1*, *bHLH39*, and *FER1* were estimated by qRT-PCR and normalized to tubulin expression. White and gray bars indicate subjective day and night, respectively.

*IRT1* expression was lengthened in mutant backgrounds in which the circadian period is lengthened. Indeed, the period of *IRT1* expression lengthened to the same extent as that of the known clock gene, *CCA1*, in two mutant backgrounds, *ztl-4* and *prr7 prr9*, known to alter period length (Fig. 2; Somers et al., 2000; Farré et al., 2005; Salomé and McClung, 2005), consistent with the clock regulation of *IRT1* expression. Therefore, we conclude that the circadian clock regulates the expression of multiple genes involved in the Fe deficiency response (*IRT1* and *bHLH39*) and in Fe storage (*FER1*).

### Fe Nutrition Status Regulates the Expression of Fe Homeostasis Gene Transcription

The expression of many Fe homeostasis genes responds to Fe nutrition status. For example, genes associated with Fe acquisition (e.g. *IRT1* and *bHLH39*) are commonly induced during Fe starvation (Vert et al., 2002; Colangelo and Guerinot, 2004; Long et al., 2010), whereas genes associated with Fe storage (e.g. *FER1*) are induced during Fe sufficiency (Gaymard et al., 1996; Petit et al., 2001). We observed that expression of *ProIRT1:LUC* was greatly increased during conditions of Fe limitation induced by addition of the Fe chelator, ferrozine (300 $\mu$M; Fig. 3, A–C) and was robust in seedlings grown on minimal medium with no Fe supplementation (Fig. 3, A, B, and D). We also detected low-level cycling expression of *ProIRT1:LUC* during growth in conditions of Fe excess (50 $\mu$M Fe; Fig. 3, A, B, and E). Interestingly, although a population of seedlings exhibited daily peaks of expression in Fe-replete conditions, individual seedlings did not express *ProIRT1:LUC* every day. Rather, an individual seedling expressed *ProIRT1:LUC* every 2 or 3 d (Fig. 3E). We interpret this to mean that expression on a single day allows sufficient IRT1 activity to take up enough Fe to support growth for more than 1 d, and only when Fe is again limiting is *IRT1* expression renewed. Because *IRT1* expression is gated by the circadian clock, expression always peaks at the same circadian phase, so the population average, which aggregates expression patterns of multiple seedlings, shows daily expression (Fig. 3, A, B, and E).

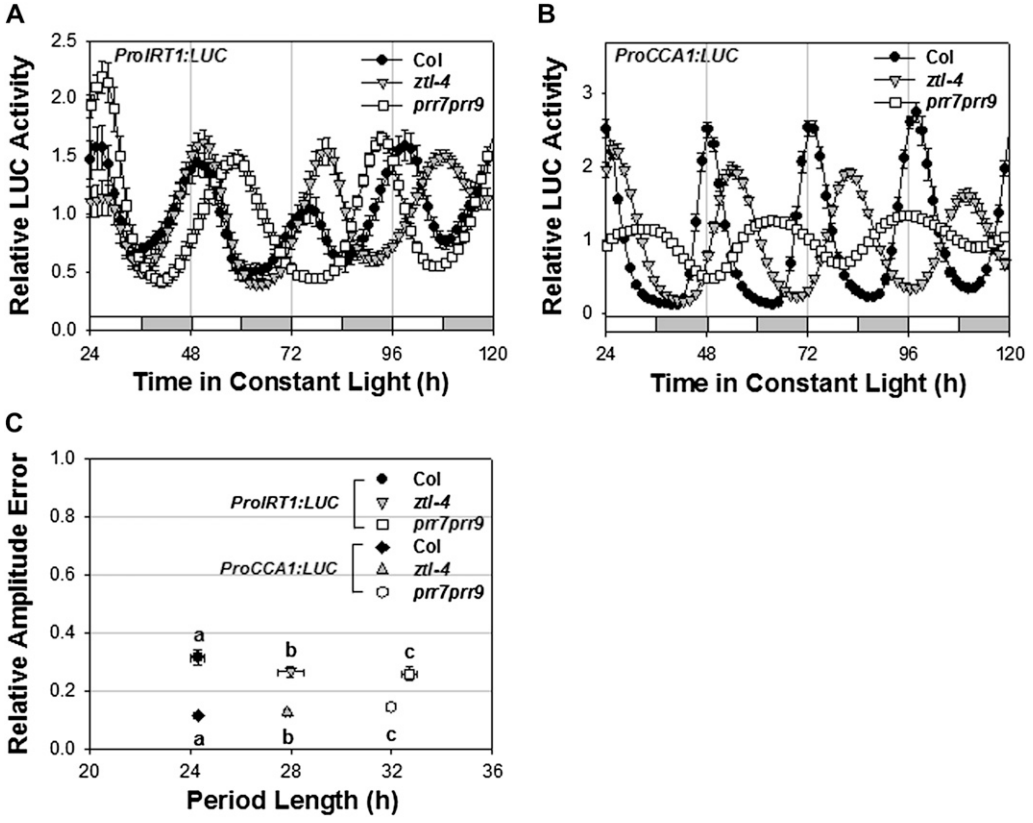


**Figure 2.** Period length of *IRT1* promoter activity is lengthened in long-period clock mutants. A and B, Seedlings of the indicated genotypes growing on minimal medium were entrained to photocycles (LD 12/12 h) for 6 d before release in LL. Average traces (mean ± SE, $n$ = 12–24) are shown, normalized to the average activity over the duration of the experiment, of *ProIRT1:LUC* (A) and *ProCCA1:LUC* (B) expression. White and gray bars indicate subjective day and night, respectively. C, Period versus relative amplitude error (RAE) of *ProIRT1:LUC* and *ProCCA1:LUC* expression. RAE is a measure of the strength of the oscillation, with RAE = 0 corresponding to a perfect sine wave and RAE = 1 defining the lower limit of statistically significant rhythmicity. Error bars represent SE. Different letters indicate significant differences ($P < 0.0001$) as determined by ANOVA.

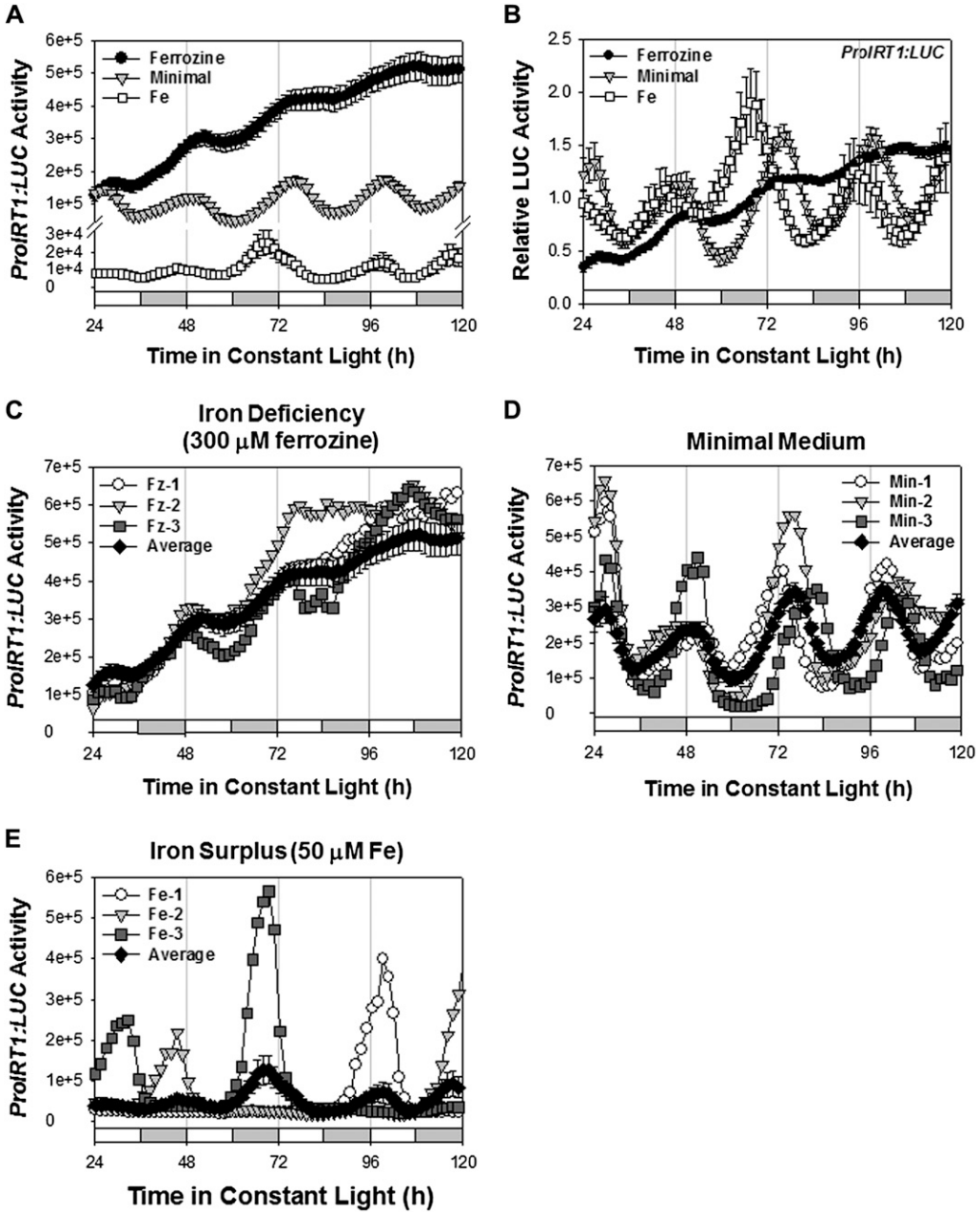


**Figure 3.** *IRT1* promoter activity under different Fe conditions. A and B, Seedlings growing on minimal medium were entrained to photocycles (LD 12/12 h) for 6 d, transferred to three different Fe conditions (300 $\mu$M ferrozine, minimal medium, and 50 $\mu$M Fe), and released in LL. Absolute (A) and relative (B) traces, normalized to the average activity over the duration of the experiment, of *ProIRT1*:*LUC* expression are shown. Data are presented as means ± SE ($n$ = 24). C to E, Traces of three individual *ProIRT1*:*LUC* seedlings and their average values under the indicated Fe conditions. White and gray bars indicate subjective day and night, respectively.

### Fe Nutrition Status Feeds Back to Regulate the Plant Circadian Clock

There is considerable interplay between the clock and the uptake and homeostasis of nutrients, including Mg, Cu, and N (Gutiérrez et al., 2008; Andrés-Colás et al., 2010; Hermans et al., 2010; Peñarrubia et al., 2010). The period of multiple clock (*CCA1* and *TOC1*) and clock-controlled (*CHLOROPHYLL a/b BINDING PROTEIN2* [*CAB2*] and *CATALASE3* [*CAT3*]) genes lengthens in response to Fe limitation induced by either chelation of Fe in the growth medium with ferrozine (300 $\mu$M) or by the inclusion of excess zinc (Zn; 50 $\mu$M), which induces Fe deficiency through competition for the high-affinity Fe uptake system (Fig. 4; Supplemental Fig. S3; Shanmugam et al., 2011). The effect of Zn is confirmed to be through competition for Fe uptake, because the period lengthening in response

to excess Zn is blocked by the provision of excess Fe. Furthermore, the period of these clock and clock-controlled genes shortens relative to the period in minimal medium in the presence of excess (50 $\mu$M) Fe. Thus, we conclude that the circadian clock responds to Fe status.

Mutants defective in Fe uptake exhibit Fe deficiency symptoms when grown in minimal medium without Fe supplementation. For example, mutants defective in *IRT1*, which encodes the high-affinity Fe(II) transporter, are chlorotic and die prior to seed set, but the chlorosis and lethality are rescued by Fe supplementation (Vert et al., 2002). Consistent with these mutants being Fe deficient on minimal medium, *ProIRT1:LUC* expression was greatly elevated in the *irt1-1* mutant (Fig. 5A). *FIT* encodes an essential bHLH transcription factor necessary for *IRT1* induction (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005; Bauer et al., 2007). Accordingly, *IRT1* expression was low in the *fit-2* mutant, even though these mutant seedlings are Fe deficient (Fig. 5A). Consistent with *irt1-1* and *fit-2* mutants being Fe deficient, the period length of both *IRT1* and *CCA1* expression was lengthened in both *irt1-1* and *fit-2* mutants (Figs. 5, B–D, and 6A). Similarly, *frd1-1* seedlings, which are defective in FRO2 ferric chelate reductase activity and Fe deficient on minimal medium (Robinson et al., 1999), also showed a lengthened period on minimal medium relative to Fe-supplemented medium (Fig. 6A; Supplemental Figs. S4 and S5). The period lengthening in each of the Fe homeostasis mutant backgrounds was seen in minimal medium and exacerbated by the addition of the Fe chelator, ferrozine (Supplemental Fig. S4). In each genotype, the long-period phenotype was rescued by the addition of Fe (50 $\mu$M) to the medium, confirming that the period lengthening was in response to Fe limitation. Thus, Fe deficiency resulting from either Fe limitation in the medium or from a genetically imposed impairment of Fe accumulation from the environment resulted in a lengthened circadian period.

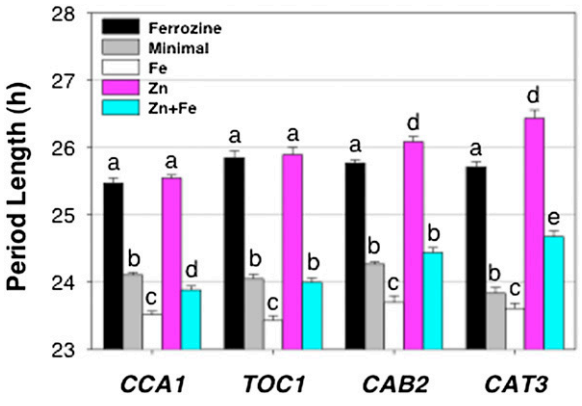


**Figure 4.** Fe status specifically changes the period length of clock gene promoter activity. Seedlings ($n$ = 24) were grown on minimal medium and entrained to photocycles (LD 12/12 h) for 6 d, transferred to different metal ion conditions (300 $\mu$M ferrozine, minimal medium, 50 $\mu$M Fe, 50 $\mu$M Zn, or 50 $\mu$M Zn plus 50 $\mu$M Fe), and released in LL. Period data of *ProCCA1:LUC*, *ProTOC1:LUC*, *ProCAB2:LUC*, and *ProCAT3:LUC* are presented as means ± SE. Different letters indicate significant differences ($P < 0.004$) as determined by ANOVA (comparisons are of the effect of growth conditions on period length as measured within each transgene, and not among different genes; thus, periods measured on different growth media with *CCA1* are compared with one another and periods measured on different growth media with *TOC1* are compared with one another, but periods measured with *CCA1* are not compared with those measured with *TOC1*).

We also examined the effects of perturbation of Fe homeostasis via the loss of leaf ferritin in the *fer1 fer3 fer4* triple mutant (Ravet et al., 2009). The period of *ProCCA1:LUC* expression was lengthened in the triple mutant relative to the wild type both in seedlings grown on minimal medium and in Fe-deficient (ferrozine-treated) seedlings (Fig. 6A; Supplemental Figs. S4 and S5). We also note that the period in Fe-replete seedlings was shortened in the triple *fer1 fer3 fer4* mutant (Fig. 6A; Supplemental Figs. S4 and S5). The mutant lacks ferritin in the shoot and, as a consequence, mutant plants have higher levels of ROS (Ravet et al., 2009). ROS has been shown to alter the expression of the evening-expressed *FLAVIN-BINDING, KELCH REPEAT, F BOX1* (*FKF1*) clock-controlled gene, although not the midday-expressed *CAB2* gene (Lai et al., 2012), and we speculate that the period shortening seen in *fer1 fer3 fer4* in Fe-supplemented conditions may result from a feedback of elevated ROS on clock function. However, the mechanistic details and target(s) of this feedback remain mysterious.

To determine which circadian clock components are required for the period-lengthening response to Fe deficiency, we examined a number of single and double mutants with defective clocks (Fig. 6B; Supplemental Fig. S6). The long-period *ztl-4* mutant (Michael et al., 2003) did not respond to Fe status and exhibited statistically indistinguishable, albeit long, periods in minimal, Fe-deficient (+ferrozine) and Fe-sufficient (+Fe) media. In contrast, the long-period *prr7 prr9* double mutant (Farré et al., 2005; Salomé and McClung, 2005) responded to Fe deficiency with lengthened period. Similarly, the *prr5-1* mutant, which exhibits only a slight shortening of circadian period length, responded to Fe deficiency with a lengthened period. The short-period *cca1 lhy* double mutant did not respond to Fe status and exhibited statistically indistinguishable, albeit short, periods in minimal, Fe-deficient (+ferrozine) and Fe-sufficient (+Fe) media. This loss of sensitivity to Fe in circadian period was seen both in the Wassilewskija (Ws) background with the *cca1-11 lhy-21* double mutant (Hall et al., 2003; data not shown) and in the Col-0 background, in which the *cca1-1* allele, originally isolated in the Ws background (Green and Tobin, 1999), had been introgressed into the *lhy-20* mutant in Col-0 through five generations of backcrossing. These data implicate CCA1, LHY, and ZTL as circadian clock targets of the Fe deficiency signal.

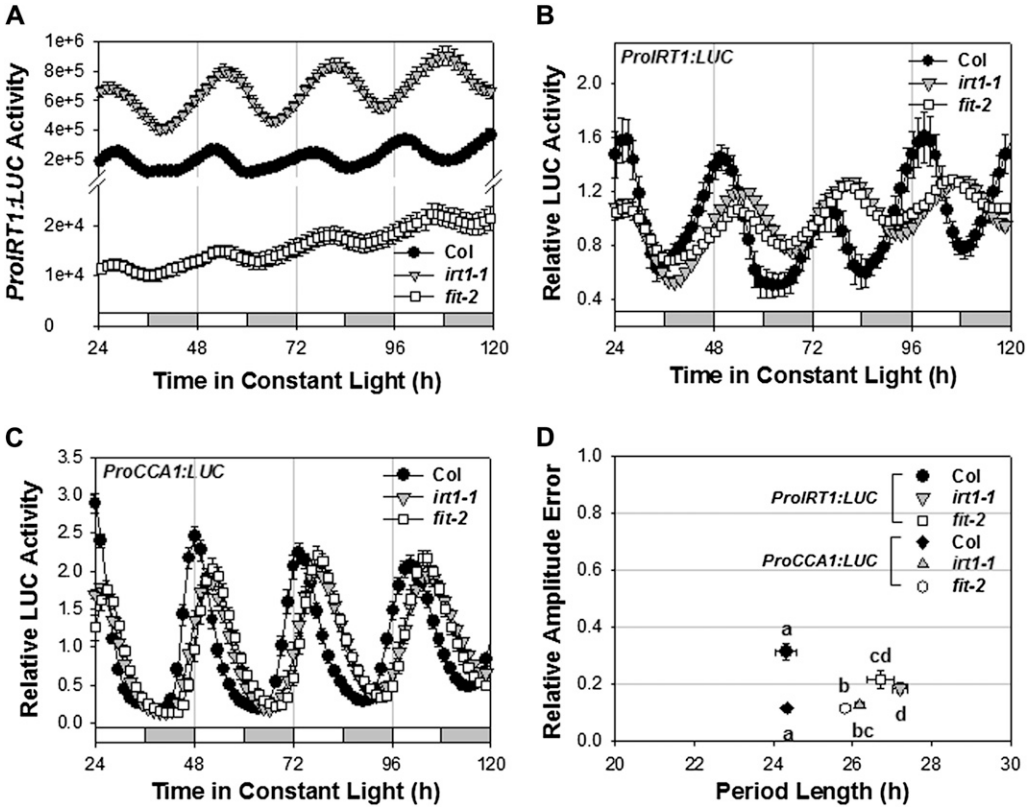


**Figure 5.** Circadian period is lengthened in Fe deficiency mutants. A to C, Seedlings of the indicated genotypes growing on minimal medium were entrained to photocycles (LD 12/12 h) for 6 d before release in LL. Absolute (A) and relative (B) traces, normalized to the average activity over the duration of the experiment, of *ProIRT1:LUC* expression ($n = 24$) and relative traces of *ProCCA1:LUC* expression (C; $n = 12$) are presented as means ± SE. White and gray bars indicate subjective day and night, respectively. D, Period versus relative amplitude error (RAE) of *ProIRT1:LUC* and *ProCCA1:LUC*. RAE is a measure of the strength of the oscillation, with RAE = 0 corresponding to a perfect sine wave and RAE = 1 defining the lower limit of statistically significant rhythmicity. Different letters indicate significant differences in period length ($P < 0.025$) as determined by ANOVA.

## DISCUSSION

The circadian clock exerts pervasive control of gene expression (Michael et al., 2008b) and thereby regulates many aspects of physiology and metabolism, including photosynthetic carbon assimilation (Hennessey and Field, 1991; Dodd et al., 2004), utilization of stored photosynthate (Graf and Smith, 2011; Stitt and Zeeman, 2012), and growth (Nozue et al., 2007; Nusinow et al., 2011; Yazdanbakhsh et al., 2011). Circadian regulation of growth suggests that there should be concomitant oscillations in water and solute fluxes (Haydon et al., 2011). Data have accumulated supporting circadian oscillations in fluxes of several molecules, including carbon into sugar and from starch (Dodd et al., 2004; Stitt and Zeeman, 2012), inorganic macronutrients (e.g. N, sulfur, $K^+$; Gutiérrez et al., 2008; Haydon et al., 2011) and micronutrients (e.g. Mg and Cu; Andrés-Colás et al., 2010; Hermans et al., 2010; Peñarrubia et al., 2010), and the key signaling molecule $Ca^{2+}$ (Dodd et al., 2006; Xu et al., 2007; Haydon et al., 2011). Moreover, some of these cycling solutes have been shown to serve as feedback regulators of clock function. For example, levels of sugars (Bläsing et al., 2005; James et al., 2008; Dalchau et al., 2011), Cu (Andrés-Colás et al., 2010; Peñarrubia et al., 2010), Mg (Hermans et al., 2010), and N (Gutiérrez et al., 2008) each affect clock function.

We have uncovered a reciprocal relationship between the micronutrient Fe and the circadian clock. The circadian clock regulates the transcription and transcript accumulation of the *IRT1* gene encoding the high-affinity Fe(II) transporter responsible for Fe uptake from the soil (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002) as well as of a gene, *bHLH39*, encoding a key transcription factor in the Fe deficiency response (Wang et al., 2013). In addition, we describe circadian regulation of *FER1*, which encodes a key Fe storage protein, ferritin (Gaymard et al., 1996). This establishes broad circadian control of the Fe homeostasis gene network. Moreover, we show that Fe status feeds back to regulate clock function, because circadian period lengthens during Fe deficiency. Such feedback regulation in which the clock regulates Fe homeostasis while Fe status feeds back to modulate clock function has also been

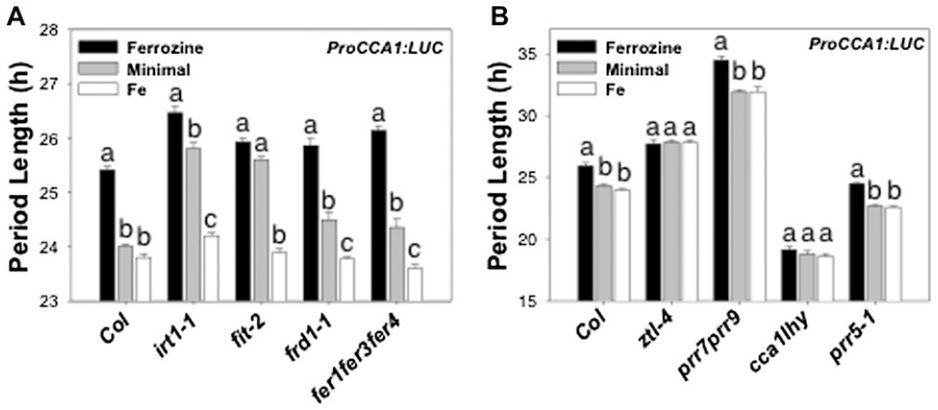


**Figure 6.** The response in circadian period to Fe status is altered by mutations affecting Fe homeostasis or circadian clock function. Seedlings ($n$ = 12) growing on minimal medium were entrained to photocycles (LD 12/12 h) for 6 d, transferred to different Fe conditions (300 $\mu$M ferrozine, minimal medium, and 50 $\mu$M Fe), and released in LL. The period of *ProCCA1:LUC* in Fe homeostasis (A) or clock-related mutants (B) is shown. Data are presented as means ± SE. Different letters indicate significant differences ($P < 0.013$) as determined by ANOVA (comparisons are of the effect of growth conditions on period length as measured within each mutant, and not among different mutants; thus, periods measured on different growth media with *irt1-1* are compared with one another and periods measured on different growth media with *fit-2* are compared with one another, but periods measured with *irt1-1* are not compared with those measured with *fit-2*).

observed in *Drosophila melanogaster* (Mandilaras and Missirlis, 2012). Down-regulation of clock-neuron expression of an Fe storage ferritin gene or of an Fe-carrying transferrin gene disrupted circadian rhythmicity in *D. melanogaster* (Mandilaras and Missirlis, 2012). In mice, there is a reciprocal regulation of the biosynthesis of heme, the important Fe-binding porphyrin, and the circadian clock, although Fe status has not been directly implicated (Kaasik and Lee, 2004). Heme levels in mice cycle with circadian period (Kaasik and Lee, 2004), and heme binding to the orphan nuclear receptor Rev-erb$\alpha$, a critical negative component of the mammalian circadian clock, regulates its interaction with a nuclear receptor corepressor complex, thereby affecting broad patterns of gene expression (Yin et al., 2007). However, this interaction of the clock with heme in mice is distinct from the interaction of the clock with Fe homeostasis in fruit flies. Although the expression of heme biosynthetic and degradative genes is under robust circadian control in *D. melanogaster* heads (Ceriani et al., 2002), down-regulation of multiple *D. melanogaster* heme biosynthetic genes did not disrupt circadian rhythmicity (Mandilaras and Missirlis, 2012).

In plants, CCA1 is emerging as a critical hub in the circadian network that is the target for nutrient status input to the circadian clock. For example, the amplitude of *CCA1* and *LHY* transcript cycling increased in response to the addition of Cu and decreased in response to Cu chelation (Andrés-Colás et al., 2010). Mg chelation increased *CCA1*, *LHY*, and *PRR9* expression (Hermans et al., 2010). Systems analysis established *CCA1* as a critical hub in the N-responsive gene network, and N metabolites were shown to shift circadian phase as monitored by *CCA1* transcription (Gutiérrez et al., 2008). In this study, we have shown that the period lengthening in response to Fe deficiency was abolished in the *cca1 lhy* double mutant, establishing a requirement for either CCA1 or LHY, or both, in the response of the clock to Fe status. A second clock mutant, *ztl-4*, also was defective in lengthening period in response to Fe deficiency, which implicates ZTL in the clock response to Fe deficiency. ZTL is not believed to directly regulate either CCA1 or LHY, which may suggest a second route of Fe signaling to the clock, independent of these two key transcription factors. ZTL is an F-box protein that regulates the proteasomal degradation of two important clock proteins, TOC1 and PRR5 (Más et al., 2003; Kiba et al., 2007; Fujiwara et al., 2008), both of which are regulators of *CCA1* and *LHY* expression (Nakamichi et al., 2010; Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2012). Thus, it is possible that the expression of *CCA1* and *LHY* is the ultimate target of Fe status signaling via ZTL.

## MATERIALS AND METHODS

### Plant Mutant Genotypes

The following Arabidopsis (*Arabidopsis thaliana*) mutant genotypes were used: *fer1 fer3 fer4* (Ravet et al., 2009), *fit-2* (Colangelo and Guerinot, 2004), *frd1-1* (Yi and Guerinot, 1996), *irt1-1* (Vert et al., 2002), *prr7-3 prr9-1* (Salomé and McClung, 2005), *prr5-1*, and *ztl-4* (Michael et al., 2003) in the Col-0 background; *cca1-1 lhy-20*, in which the *cca1-1* allele was originally isolated in the Ws background (Green and Tobin, 1999), was introgressed into a *lhy-20* mutant in the Col-0 background (Michael et al., 2003) through five sequential backcrosses; and *cca1-11 lhy-21* (Hall et al., 2003) in the Ws background.

### Generation of Constructs and Transgenic Plants

Firefly luciferase was driven from Arabidopsis clock and clock-controlled gene promoters, including *ProCCA1:LUC* and *ProLHY:LUC* (Salomé and

McClung, 2005), *ProTOC1*:*LUC* (Michael and McClung, 2002), *ProCAB2*:*LUC* (Millar et al., 1992), and *ProCAT3*:*LUC* (Michael and McClung, 2002). Promoters of *IRT1* (−1,086 to −63, where the A of the ATG start codon = +1), of *bHLH39* (−755 to −1), and of *FER1* (−1,394 to −5) were amplified from Col-0 genomic DNA using gene-specific primers (Supplemental Table S1). The amplified products were cloned into pCR8/GW/TOPO (Invitrogen) and subcloned into pZPΩLUC+ (Schultz et al., 2001). The resulting binary vectors were introduced into *Agrobacterium tumefaciens* strain AGL1 by electroporation, and *ProbHLH39*:*LUC* and *ProFER1*:*LUC* were transformed into Col-0 (Bechtold et al., 1993). *ProIRT1*:*LUC* was initially transformed into Col-*gl1* and moved into the Col-0 wild-type background by genetic crossing. With the exception of *fer1 fer3 fer4*, *Pro*:*LUC* transgenes were introduced into the mutants via genetic crossing and selection of F2 plants with the appropriate morphological and/or circadian phenotype in LL after LD entrainment. *fer1 fer3 fer4* plants were transformed by infiltration via *A. tumefaciens* (Bechtold et al., 1993).

## Bioluminescence Assay

Rhythm assays were performed as described (Salomé and McClung, 2005) except that seeds were sterilized, stratified in the dark at 4°C for 3 d, and sown on minimal medium consisting of 2 mM $Ca(NO_3)_2$, 0.75 mM $K_2SO_4$, 0.65 mM $MgSO_4$, 0.1 mM $KH_2PO_4$, 10 $\mu$M $H_3BO_3$, 0.1 $\mu$M $MnSO_4$, 0.05 $\mu$M $CuSO_4$, 0.05 $\mu$M $ZnSO_4$, 0.005 $\mu$M $(NH_3)6Mo_7O_{24}$, 0.5 g of MES, and 0.5% Suc (w/v), adjusted to pH 6.0 and solidified with 0.7% type M agar (Marschner et al., 1982; Yi and Guerinot, 1996). Seedlings were entrained for 6 d in photocycles (LD 12/12 h) before transfer to LL for LUC activity measurement using a Packard TopCount Luminometer. Fe deficiency was imposed by the addition of the Fe chelator ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate (HACH Chemical)] to the minimal medium at 300 $\mu$M (Yi and Guerinot, 1996). Fe repletion was imposed through the addition of 50 $\mu$M Fe(III)-EDTA to the minimal medium (Yi and Guerinot, 1996). Rhythms were analyzed by fast Fourier transform nonlinear least-squares (Plautz et al., 1997). Whole-seedling LUC imaging was performed using an ORCA II ER CCD camera (C4742-98 ERG; Hamamatsu Photonics; http://www.hamamatsu.com), with data collected at the time of peak expression for each transgene (Fig. 1, A and B) with 60-min exposure times. Images were analyzed with MetaMorph software (Molecular Devices; http://www.moleculardevices.com/Products/Software/Meta-Imaging-Series.html).

## Expression Analysis by qRT-PCR

Seedlings were entrained for 10 d in photocycles (LD 12/12 h) and transferred to LL. Samples were collected every 4 h for the following 3 d. RNA was extracted using the Qiagen RNeasy Plant Mini Kit. First-strand complementary DNA synthesis used 2 $\mu$g of total RNA with the SuperScript III first-strand synthesis system (Invitrogen). The complementary DNA was diluted 10 times with water, and 1 $\mu$L was used for PCR amplification using a SYBR Premix Ex Taq II (Takara) with gene-specific primers (Supplemental Table S1). mRNA abundances were calculated using the comparative cycle threshold method, with *TUB3* (At5g62700) as the normalization control.

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this study are as follows: *bHLH39* (At3g56980), *CAB2* (At1g29920), *CAT3* (At1g20620), *CCA1* (At2g46830), *CCR2* (At2g21660), *CHE* (At5g08330), *FER1* (At5g01600), *FER3* (At3g56090), *FER4* (At2g40300), *FIT* (At2g28160), *FRD1*/*FRO2* (At1g01580), *GI* (At1g22770), *IRT1* (At4g19690), *LHY* (At1g01060), *PRR5* (At5g24470), *PRR7* (At5g02810), *PRR9* (At2g46790), *TIC* (At3g22380), *TOC1* (At5g61380), and *ZTL* (At5g57360).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1**. Diurnal and circadian regulation of Fe homeostasis gene expression.

**Supplemental Figure S2**. Temporal and spatial expression of Fe homeostasis gene expression.

**Supplemental Figure S3**. Fe status specifically changes period length of clock gene promoter activity.

**Supplemental Figure S4**. Circadian period is lengthened in Fe homeostasis mutants.

**Supplemental Figure S5**. Circadian period is lengthened in Fe homeostasis mutants.

**Supplemental Figure S6**. The response in circadian period to Fe status is altered by mutations affecting Fe homeostasis or circadian clock function.

**Supplemental Table S1**. Oligonucleotide primers used in this study.

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